The Relationship of the Australian Freshwater Crayfish Genera *Euastacus* and *Astacopsis*

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(Communicated by J.R. Merrick)

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Lawler, S.H. and Crandall, K.A. (1998). The relationship of the Australian freshwater crayfish genera *Euastacus* and *Astacopsis*. *Proceedings of the Linnean Society of New South Wales* **119**, 1–8.

The genera *Euastacus* and *Astacopsis* are thought to be closely related because of their similar morphologies and ecologies as well as their location in southeastern Australia. Members of the genus *Astacopsis* are restricted to Tasmania whereas *Euastacus* ranges from northern Cape York to southern Victoria. In order to test for the monophyly of each genus and to examine the evolutionary relationships among genera, DNA sequences from the 16S region of the mitochondrial rDNA array from members of these two genera were compared. Our data indicate that the genera are evolutionarily distinct. *Astacopsis* appears to be paraphyletic, with members of the genus *Euastacus* forming a monophyletic group within the *Astacopsis*.

Manuscript received 21 August 1997, accepted for publication 5 January 1998.

Keywords: *Euastacus*, *Astacopsis*, freshwater crayfish, phylogeny, mitochondrial DNA, maximum likelihood.

INTRODUCTION

The genera *Euastacus* and *Astacopsis* are both called spiny crayfish and are known to prefer cool, pristine freshwater habitats. The genus *Euastacus* is widespread in eastern mainland Australia, while *Astacopsis* is restricted to Tasmania. Both genera have been recently revised. The genus *Astacopsis* is now thought to contain three species (Hamr 1992), although in the past as few as two were recognised (Swain et al. 1982) and as many as four (Riek 1969). The genus *Euastacus* is much larger and variable with 41 species currently recognised (Morgan 1986, 1988, 1989, 1997). According to Riek (1972), the two genera are sister taxa and their closest relative is the genus *Astacoides*, which is restricted to Madagascar.

The spiny crayfishes have much in common both morphologically and ecologically. Species distributions closely correspond to river drainages, with high endemism throughout southeast Australia (Merrick 1993, 1995). Clark (1936) divided the two genera based on the telson (membranous in *Euastacus* and calcareous in *Astacopsis*), the stems of podobranchs (wing-like in *Euastacus*) and the relative spininess of the abdomen. Nevertheless, the monophyly of the genus *Astacopsis* has been questioned (Horwitz 1996), reflecting a long-standing concern that the two genera do not form natural species groups.

Many of the morphological characters used in taxonomy and phylogeny of these species are highly variable or subject to convergent evolution. Attempts to divide species of *Astacopsis* based on spininess found that this character was influenced by both the habitat and geographic region in which the animals were collected (Swain et al. 1982).

Since the ecological requirements of these genera are virtually identical, they may have similar morphologies for reasons other than taxonomic relationship. Convergent evolution in morphological characters is known to occur in many crayfish groups (Hobbs 1974).

Australian freshwater crayfish can be broadly separated into true burrowers, which hold their chelae in a vertical plane, and the moderate burrowers, whose chelae are horizontal. The moderate burrowers include the genera *Euastacus*, *Astacopsis*, *Euastacoides, Astacoides, Cherax* and *Paranephrops* (Riek 1972). Of these, *Astacoides* is presumed to be the most closely related (Riek 1972), but it is only found in Madagascar and tissue was not available. *Paranephrops* occurs only in New Zealand, and samples were unavailable for analysis. Thus the genus chosen as an outgroup was *Cherax,* primarily because it is phylogenetically distinct from *Euastacus* and *Astacopsis* and because multiple species were available, allowing a comparison of genetic diversity among genera.

Others have attempted to identify natural phylogenetic groups within the freshwater crayfish using molecular characters. Patak and Baldwin (1984) examined the relationships of 6 freshwater crayfish genera using distances generated using antibody/antigen reactions and electrophoretic domains of the blood protein haemocyanin. The genera *Euastacus* and *Astacopsis* could not be distinguished using this approach, although the genus *Cherax* was found to be genetically distinct and basal to the other two genera. However, using data from 30 allozyme loci from 7 species of *Euastacus* and all 3 species of *Astacopsis*, Avery and Austin (1997) found more differences between the genera than between species within each genus, supporting their current taxonomic status.

This study, based on DNA sequences from the 16S mitochondrial region, tests whether *Astacopsis* is distinct from *Euastacus* and provides a preliminary look at relationships among species within these two genera using the genus *Cherax* as an outgroup.

TABLE 1

Species used and the location from which they were collected.

Species	Location
Astacopsis franklinii	New Town Rivulet, Northern Hobart, Tasmania
A. tricornis	Huon River, Western Tasmania
Cherax cuspidatus	Bell Creek Rd., Caloundra, Queensland
C. destructor albidus	Barney Creek south of Halls Gap, Victoria
C. robustus	Crayhaven Yabbie Farm, North Arm Cove, NSW
Euastacus armatus	Hoy River, Harrietville, Victoria
E. australasiensis	Wirreanda Creek, north of Church Point, NSW
E. bispinosus	Burrong Falls off of Rose Creek Rd., Victoria
E. yarraensis	Upper Gellibrand River, west of Barramunga, Victoria

MATERIALS AND METHODS

Crayfish of the genera *Euastacus, Astacopsis* and *Cherax* were collected from locations throughout eastern Australia, usually by turning rocks and catching individuals by hand (see Table 1). The genus *Cherax* was included in the study as an outgroup because this genus is clearly phylogenetically distinct from *Euastacus* and *Astacopsis,* and because *Cherax* has been hypothesized to be most closely related to *Euastacus* (Riek 1969; Crandall et al. 1995; Patak and Baldwin 1984).

Tissue from the gills and tail were frozen or preserved in ethanol. DNA was extracted and the 16S region of the mitochondria was amplified via the polymerase chain reaction using standard protocols (Crandall et al. 1995). PCR reaction conditions consisted of an initial two minute denaturation at 92°C, followed by 30 cycles of one minute denature at 92°C, 30 second annealing at 45°C, and 30 second extension at 72°C. These 30 cycles were then followed by a 10 minute extension at 72°C. PCR products were sequenced from both ends with an ABI 377 automated sequencer, following the manufacturer's instructions. Finally, the sequences were edited and spliced together by eye to make a single contiguous ~520 base pair unit. Sequences were aligned using Clustal W (Thompson et al. 1994).

Unrooted phylogenies, with *Cherax* specified as the outgroup, were estimated using the maximum parsimony approach with equal weights assigned to all changes (PAUP*, 4.0d56: Swofford 1997). Weighting schemes that incorporated the observed transition bias had no effect on the tree topology. An exhaustive search was performed which examines every possible tree topology (PAUP*, 4.0d56: Swofford 1997). Phylogenetic relationships were also estimated using maximum likelihood (Felsenstein, 1981) and neighbor-joining (Saitou and Nei 1987) as implemented by PAUP* (Swofford 1997). The model of evolution used in these analyses was determined by the procedure outlined in Huelsenbeck and Crandall (1997); namely, a likelihood ratio test was used to determine significant differences among models of evolution. The likelihood ratio statistic, $l = -2(\ln L_0 - \ln L_1)$, was compared to a χ^2 distribution with a Bonferroni adjusted significance level for multiple comparisons. Phylogenetic signal was measured via the g_1 statistic (Hillis and Huelsenbeck 1992).

Relative amounts of genetic diversity in the different genus lineages was measured using the approach of Crozier (1992), as implemented by the computer program Conserve 3.0 (Agapow 1997). Branch lengths were estimated as the proportion of overall diversity within the phylogeny (Crozier and Kusmierski 1994).

Confidence in resulting clades was assessed using the bootstrap procedure (Felsenstein 1985). The testing of alternative phylogenetic hypotheses was performed using a sign test (Crandall and Fitzpatrick 1996) which is an unweighted version of the Wilcoxon signed rank test (Templeton 1983).

RESULTS

The resulting sequences have been deposited in Genbank under accession numbers AF044240–AF044248. The exhaustive parsimony search resulted in a single most parsimonious tree (Fig. 1). The g_1 statistic showed significant skewness in the tree distribution (–0.8575) indicating significant phylogenetic signal ($P < 0.01$, Hillis and Huelsenbeck 1992). This phylogenetic signal remained even after constraining clades with high bootstrap support.

Several molecular evolutionary hypotheses were tested using these sequence data, as shown in Table 2. Firstly we rejected the hypothesis of equal base frequencies as our data show an A/T bias (A = 0.322, T = 0.353, C = 0.108, and G = 0.217). Likewise, equal rates of transitions and transversions were rejected, with these sequences showing a transition/transversion ratio of 2.023 (Table 2). We were also able to reject the hypothesis of equal evolutionary rates among sites, since incorporating a model with the gamma distribution increased the likelihood over the null hypothesis of equal rates among sites (G shape parameter = 0.384, Table 2). Incorporating the proportion of invariant sites into the model does not significantly increase our likelihood. Thus we concluded that the most appropriate model of evolution for our data was the HKY85+G model (for details, see Swofford et al. 1996). Using this model of evolution we estimated the maximum likelihood tree to be identical to the parsimony tree (Fig. 1). The same tree was also estimated using the neighbor-joining method.

TABLE 2

Test of molecular hypotheses to determine the appropriate model of evolution for maximum likelihood and neighbor-joining searches. The Bonferroni adjusted significance level for five comparisons is a = 0.01.

Null Hypothesis	Models Compared	-lnL0	-lnL1	-2lnλ	d.f.	*P*
Equal base frequencies	H_0: JC69 H_1: F81	1957.46	1904.51	105.9	3	4.14×10^{-23}
Equal transition/transversion rates	H_0: F81 H_1: HKY85	1904.51	1863.10	82.82	1	4.55×10^{-20}
Equal rates among sites	H_0: HKY85 H_1: HKY85+Γ	1863.10	1826.31	73.58	1	4.90×10^{-18}
Proportion of invariable sites	H_0: HKY85+Γ H_1: HKY85+Γ+invar	1826.31	1826.31	0	1	1
Molecular Clock	H_0: HKY85+Γc H_1: HKY85+Γ	1836.73	1826.31	20.84	7	1.57 x 10–3

The tree presented in Figure 1 indicates that the genus *Astacopsis* is paraphyletic with respect to *Euastacus*. *Euastacus* appears to be monophyletic, although the maximum likelihood bootstrap support was only 61% for this grouping. Significance of bootstrapping values is open to interpretation (Hillis and Bull 1993), so tests of these results were done using a sign test to compare several alternative phylogenetic hypotheses of *Astacopsis* and *Euastacus* monophyly and nonmonophyly (Fig. 2). The hypothesis that both *Astacopsis* and *Euastacus* are monophyletic can be rejected at $P = 0.0592$ (Fig. 2A). The hypothesis that both *Euastacus* and *Astacopsis* are both paraphyletic can be rejected at the $P = 0.0898$ level of significance (Fig. 2B). Finally, we can test the hypothesis that *Astacopsis* is monophyletic and derived from the paraphyletic *Euastacus* (Fig. 2C). We reject this hypothesis at the $P = 0.0119$ level of significance. Clearly these tests are only marginally significant. Future work will incorporate more taxa and more sequence data, including data from nuclear genes, to further test these hypotheses. Thus our sequence data supports the monophyly of *Euastacus* and the paraphyly of *Astacopsis*.

Genetic diversity was measured for each lineage. The lowest genetic diversity was found in the genus *Euastacus* (GD = 0.0924) despite having the largest number of species represented. *Astacopsis* was intermediate in genetic diversity (GD = 0.168), almost doubling the value for *Euastacus*. Finally, *Cherax* had the highest diversity (0.2269). The molecular clock test rejected the hypothesis of equal rates among all lineages, however this rejection was not highly significant (Table 2). Differences in rates of evolution would not alter the fact that the high genetic diversity in *Astacopsis* should elevate its conservation status, and the imperilment of *A. gouldi* support this point (Horwitz 1994).

DISCUSSION

Unfortunately our efforts to amplify DNA from *A. gouldi* were not successful, so all three species of *Astacopsis* are not represented in our phylogeny. Nevertheless, the data presented here suggest that the genus is evolutionarily distinct from *Euastacus*. If

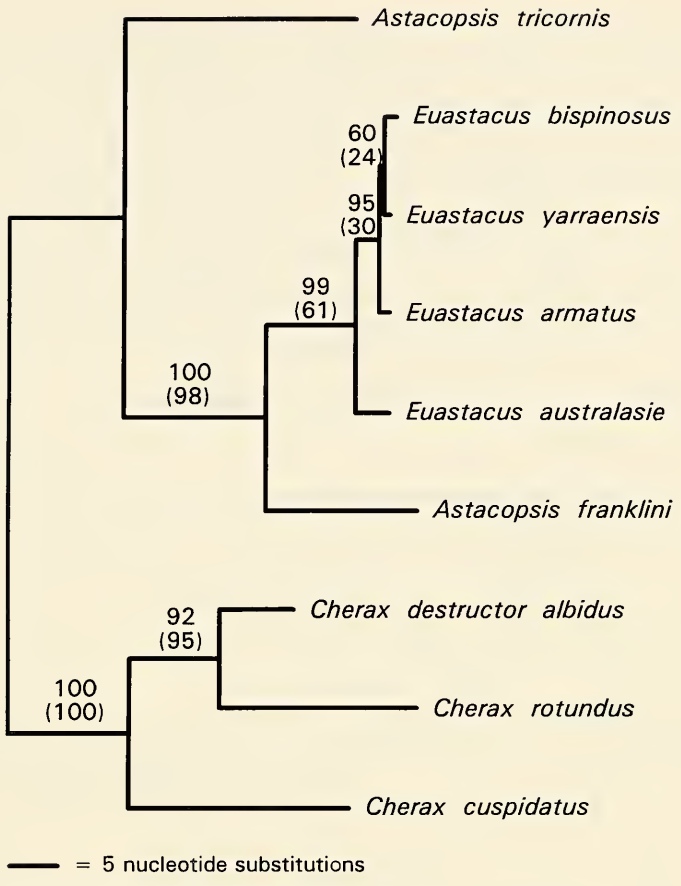

Figure 1. The maximum parsimony, maximum likelihood and neighborhood joining tree is shown with branch lengths proportional to the amount of nucleotide divergence along each branch. The scale at the bottom of the figure gives an indication of the number of substitutions occurring along each branch. The tree is unrooted with the *Cherax* species designated as the outgroup. The relative support for each clade is shown as a bootstrap percentage at the nodes with the parsimony bootstrap values shown on top and the maximum likelihood bootstrap values shown in parentheses. The bootstrap values were based on 1000 replications.

we root our phylogeny with the *Euastacus* clade (data not shown), then the *Astacopsis* are not even in the same clade as *Euastacus* and appear ancestral to *Cherax*. Our phylogeny based on two *Astacopsis* and four *Euastacus* species indicates that the mainland genus *Euastacus* is a monophyletic group within the *Astacopsis,* which is paraphyletic.

The highest genetic diversity was found in the genus *Cherax*, but the fact that *Astacopsis* has almost twice the genetic diversity of *Euastacus* supports the idea that *Astacopsis* is an older evolutionary lineage from which *Euastacus* may be recently derived. This result is especially surprising because the two *Astacopsis* species sequenced are difficult to distinguish morphologically, and were previously thought to be very closely related, even conspecific (Swain et al. 1982). Our data give the genus *Astacopsis* a high conservation priority from a genetic perspective (Crozier 1992).

Avery and Austin (1997) were unable to distinguish between *A. tricornis* and *A. franklinii* within the genus *Astacopsis*, and *E. bispinosus, E. armatus* and *E. yarraensis* within *Euastacus* using allozyme electrophoresis. Although our mitochondrial data sup-

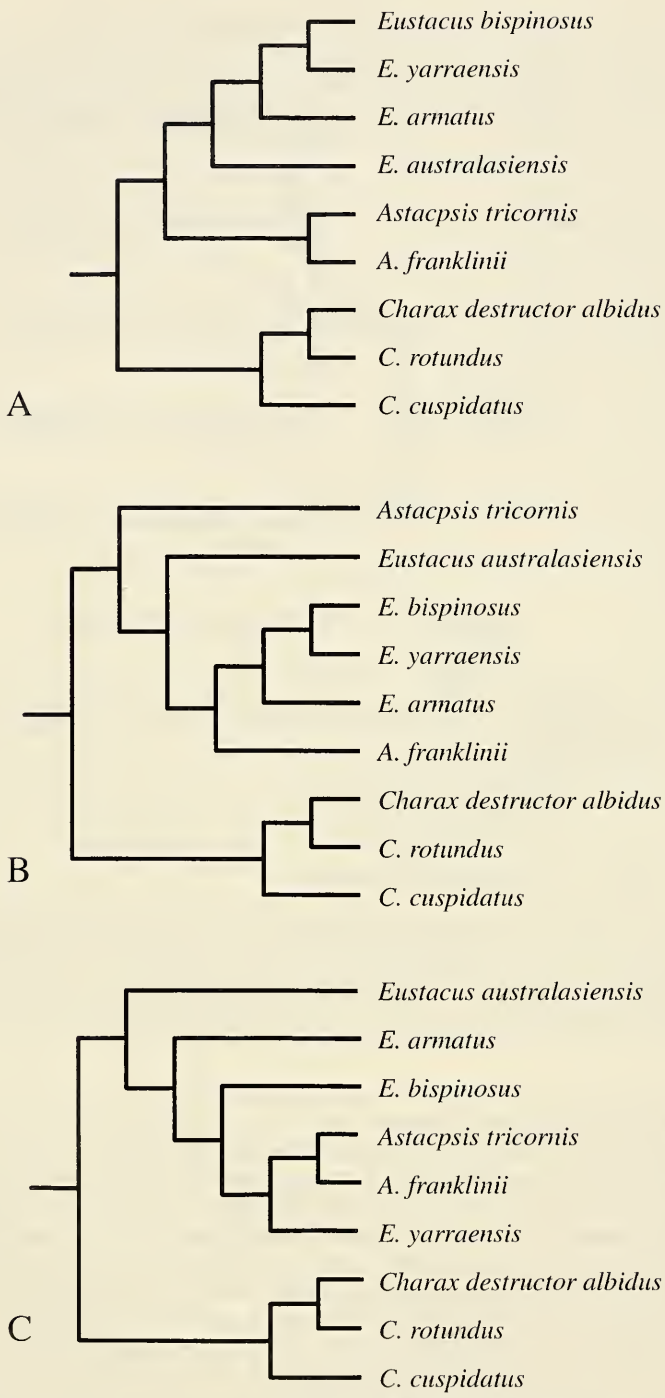

Figure 2. The alternative phylogenetic hypotheses tested. A) *Astacopsis* and *Euastacus* are monophyletic, B) *Astacopsis* and *Euastacus* are paraphyletic (this phylogeny differs from Figure 1 in the placement of *E. australasiensis* between the two *Astacopsis*, making *Euastacus* paraphyletic), and C) *Astacopsis* is monophyletic and derived from the paraphyletic *Euastacus*.

port the close relationship between the three species of *Euastacus* (notice the short branch lengths in Fig. 1), the two species of *Astacopsis* are genetically quite distinct.

The primary vicariance event for Tasmanian invertebrates seems to be the high country of central Tasmania, which would have been periglacial as recently as 7000 years ago (Mesibov 1994). Thus the major split among faunal components in Tasmania is east/west, reflecting the distribution of the species *Astacopsis tricornis* and *A. franklinii* (Hamr 1992). Our data showing that these two species are distinct support this vicariance event for the genus *Astacopsis*. *Astacopsis gouldi*, which occurs along the northern coast of Tasmania, nevertheless does not occur in the Tamar river system in northcentral Tasmania, and therefore has distinct western and eastern metapopulations (Horwitz 1994). Sequence data from these populations would be of considerable interest.

Hypotheses of relationships among members of *Astacopsis* and *Euastacus* have focused on *Euastacus* species in southeastern Victoria. The last link between Tasmania and Victoria about 12,000 years ago across Flinders Island to Wilson's Promontory (Williams 1974:171), would have allowed contact between the Tasmanian and southeast Victorian freshwater crayfish. Four species of *Euastacus* from southeastern Victoria (*E. bidawalus*, *E. diversus*, *E. neodiversus* and *E. woiwuru*) share a morphological character (the male cuticle partition) with the *Astacopsis* (Morgan 1983). Thus there may be a subset of *Euastacus* species that are more like *Astacopsis*, and sequence data from these would be of particular interest. Preliminary data (not shown) place *E. bidawalus* firmly within the *Euastacus* cluster, supporting the two genera as they stand.

Horwitz (1996) suggested that *A. franklinii* is more closely related to *Euastacus woiwuru* and *E. neodiversus* than to *A. gouldi* and *A. tricornis*, based on morphological characters in a dichotomous key. Testing of these hypotheses will require sequence data from more taxa.

In summary, this study indicates that the genus *Astacopsis* is not monophyletic, nor is it a derived branch of the genus *Euastacus*. Further research will include sequencing *A. gouldi* and more species of *Euastacus*, particularly the species that have a male cuticle partition and come from southeastern Victoria.

ACKNOWLEDGEMENTS

We thank Margie Kinnersley for providing some of the sequence data. Alistair Richardson provided tissues of *Astacopsis gouldi* and Chris Austin provided tissues of *A. franklinii*. We also thank Drs Richardson and Austin, Pierre Horwitz and Barb Mable for assistance in collecting the specimens used in this study. This work was supported by the Alfred P. Sloan Foundation, the National Science Foundation, and Deakin University (KAC).

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